Paclitaxel Induces Apoptosis via Protein Kinase A- and p38 Mitogen-activated Protein-dependent Inhibition of the Na^+/H^+ Exchanger (NHE) NHE Isoform 1 in Human Breast Cancer Cells[1]

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ABSTRACT

Purpose: The molecular signal components essential to paclitaxel-dependent apoptosis in breast cancers are potential targets for combined therapy. However, the signal mechanisms underlying paclitaxel action still need to be better defined.

Experimental Design: In a breast cancer cell line, pharmacological agents and transient transfection with dominant interfering and constitutive active mutants were used to identify the signal transduction module involved in the regulation of paclitaxel-induced apoptosis and to evaluate its potential as a therapeutic target.

Results: In MDA-MB-435 cells, paclitaxel treatment stimulated the activity of both protein kinase A and p38, and inhibited the activity of the Na^+/H^+ exchanger isoform 1 (NHE1) with similar IC_{50} concentrations as for its activation of apoptosis. Activation and inhibition experiments demonstrated that protein kinase A and p38 participate sequentially upstream of the NHE1 in regulating the paclitaxel-induced apoptotic pathway. Importantly, concurrent specific inhibition of the NHE1 with paclitaxel treatment resulted in a synergistic induction of apoptosis and a reduction in the paclitaxel IC_{50} for apoptosis. This sensitization of paclitaxel apoptotic action by specific inhibition of NHE1 was verified in breast cancer cell lines with different paclitaxel sensitivity.

Conclusions: We have, for the first time, identified NHE1 as an essential component of paclitaxel-induced apoptosis in breast cancer cells and, importantly, identified that simultaneous inhibition of the NHE1 results in a synergistic potentiation of low-dose paclitaxel apoptotic action. As specific NHE1 inhibitors have finished Phase II/Phase III clinical trials for myocardial protection, there is the possibility for a rapid biological translation of this novel therapeutic strategy to a clinical setting.

INTRODUCTION

An important aspect of modern chemotherapy is the identification of signal transduction targets that substantially increase the drug-dependent toxic effect (1, 2). In this context, one of the more interesting and important antineoplastic drug families is that of the taxanes, such as paclitaxel and docetaxel, which are active against a broad spectrum of cancers that are often refractory to other types of chemotherapy (1–4). The major cellular target for the taxanes is considered to be the tubulin/microtubule system (4). Paclitaxel binds to β-tubulin in a 1:1 stoichiometry with tubulin heterodimers stabilizing microtubules and driving a high percentage of cells to arrest in the G_2/M phase, progress slowly in the cell cycle without cytokinesis, form multinucleated polyploid cells, and undergo apoptosis.

Recent discoveries of signaling processes altered by paclitaxel suggest that it can induce apoptosis through multiple mechanisms (5). Paclitaxel is known to modulate PKA[3] (6–9) and the various MAP kinases (10–16) in normal and cancer human breast cells. The heterogeneity of the mechanisms observed in these studies suggests that coordinated and reciprocal alterations in the activities of various kinases must be important aspects of the apoptotic response to paclitaxel and that, furthermore, the specific set of kinases used for the apoptotic response is probably tissue- or tumor-specific (16). However, until now the studies elucidating the modulation and probable role of various signal transduction components in paclitaxel action have been disjointed, often without an indication of the possible interactions between the systems thus far identified. In particular, whereas the MAP kinase and PKA pathways have been

Received 9/27/02; revised 12/20/02; accepted 1/23/03.
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[1] Supported by grants from the Italian Ministry of Health (RF95-193), the Consiglio Nazionale delle Ricerche Grant 99.02574.CT04 (to S. J. R.), and by CEGBA (Centro di Eccellenza di Genomica in Campo Biomedico ed Agrario). A. B. was supported by a fellowship from the Italian Association for Cancer Research (AIRC).
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[3] The abbreviations used are: PKA, protein kinase A; MAP, mitogen-activated protein; NHE1, Na^+/H^+ exchanger isoform 1; DMA, 5-(*N*,*N*-dimethyl) amiloride; p38, p38 mitogen-activated protein kinase; MTT, methylthiazoletetrazolium; pHi, intracellular pH; dn, dominant-negative; Fsk, forskolin.

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examined in several studies, no clear interaction has been yet been established between them for paclitaxel-induced apoptosis.

Inhibition of the NHE1 has been shown to be an early signal transduction event that may participate either directly or indirectly in the regulation of the apoptotic response by many drugs (17–20). NHE1 is an ubiquitously expressed cell membrane protein that contributes to the regulation of pHi homeostasis, and seems to be particularly important in transformation (20–23) and in tumor cell biology (20, 24, 25). Recent evidence demonstrating a direct regulation of NHE1 activity by the cytoskeleton (23, 26) and a reciprocal ability of NHE1 to regulate cytoskeletal dynamics (27) provides a structural rationale for a role of the NHE1 in paclitaxel-dependent apoptosis. However, its involvement in this process is still unknown.

In the present study we describe a signal transduction module involved in the induction of apoptosis by paclitaxel in a human metastatic breast cancer cell line, MDA-MB-435 (28–30). A metastatic cell line was chosen, because paclitaxel has been primarily used for treatment of metastatic disease (31). We report here that the inhibition of NHE1 plays a fundamental role in paclitaxel-dependent induction of apoptosis through a mechanism that depends directly on the activation of PKA and p38α MAP kinase. Targeted down-regulation of NHE1 activity with a specific inhibitor synergistically potentiated the paclitaxel-dependent apoptotic response at very low paclitaxel concentrations. This fundamental role of the NHE1 in paclitaxel-driven apoptosis was corroborated in other breast cancer cell lines of differing neoplastic state and receptor status. Importantly, specific inhibitors of the NHE1 isoform have completed Phase II/Phase III clinical trials (32–34), thus providing the possibility for its inclusion in clinical trials with paclitaxel to chemopotentiate its therapeutic action.

MATERIALS AND METHODS

Cells and Expression Vectors. MDA-MB-435 cells, a human breast tumor cell line derived from a pleural effusion of a malignant human tumor (28, 29) and two human breast tumor cell lines derived from nonmalignant tumors, MCF-7 (35) and SKBR3 (36), were cultured as described previously (25, 30). Plasmids for the dn p38α (K>M) and the constitutively active (ca)MKK6(E) in the KRSPA expression vector were obtained from Dr. Stephan Ludwig of the University of Würzburg, Würzburg, Germany. Transient transfections were performed with the LipoTaxi reagent (Stratagene) according to the manufacturer's instructions. Experimental treatments were started 24 h after transfection.

pHi and NHE Activity Determination. Cytoplasmic pHi was measured spectrofluorimetrically at 37°C with the fluorescent pH sensitive probe, 2′, 7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (pentaacetoxymethy) ester, trapped intracellularly in cell monolayers grown on glass as described previously (21, 25). The activity of the Na^+/H^+ exchanger was measured by monitoring pHi recovery after an intracellular acid load produced with the NH_4Cl prepulse technique (37). Preliminary experiments demonstrated that ~95% of the sodium-dependent pHi recovery is inhibited by the specific NHE1 inhibitor, DMA.

PKA Assay. After treatment for the indicated times and concentrations, monolayers were washed twice with cold PBS, scraped into ice-cold homogenization buffer [5 nM EDTA, 10 mM EGTA, 50 mM 2-mercaptoethanol, 1 mM phenylmethanesulfonyl fluoride, 10 mM benzamidine, and 50 mM Tris (pH 7.5)], subjected to sonification, and the homogenate centrifuged 30 min at 15,000 × *g* at 4°C. Protein content of the supernatant was measured by the Bradford method. PKA activity was evaluated by measuring the cyclic AMP-dependent phosphorylation of an immobilized peptide substrate (RFARKGSLRQKNV) in an ELISA assay according to the manufacturer's (MBL, Nagoya, Japan) instructions. Pharmacological stimulation of the endogenous PKA by Fsk was used as a positive control.

Phospho-MAP Kinase Assays and NHE1 Analysis by Western Blot. Cells were grown in 10-cm culture dishes and treated with different concentrations of paclitaxel for the indicated times. For the kinase activation assays total cellular protein was extracted in SDS-sample buffer [50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 0.1% bromphenol blue], and ~50 μg was separated on 10% SDS-PAGE and transferred to Immobilon P (Millipore). Activated kinases were detected using antiphosphokinase antibodies and corrected for expression using total kinase antibodies (New England Biolabs, Beverly, MA). Western blots were visualized by enzyme-linked chemiluminescence after sequential incubations with horseradish peroxidase-conjugated goat antirabbit IgG for 30 min, three washes with PBS, incubation with the substrate luminol, and subsequent development. The level of expression of NHE1 measured by Western blot as described previously (21, 25).

Cell Death ELISA. Cells were plated at 5×10^5 cells/well in a 96-well microtiter plate and grown for 24 h. The cells were transfected with the gene of interest or its empty vector. After 24 h, the cells were treated experimentally, and samples harvested and analyzed for histone-associated DNA fragments as per the manufacturer's instructions in the Cell Death ELISA^{PLUS} kit (Roche Molecular Biochemicals, Milan, Italy). The test is based on the detection of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates by biotinylated antihistone-coupled antibodies, and their enrichment in the cytoplasm is calculated as absorbance of sample cells/absorbance of control cells. Enrichment factor was used as a parameter of apoptosis and shown on the Y-axis as mean ± SE. The increase in apoptosis was verified in random samples by measuring the amount of DNA laddering (data not shown).

RESULTS

Paclitaxel Induces Apoptosis in MDA-MB-435. We first verified whether treatment with paclitaxel might differentially affect cellular proliferation and/or apoptosis with dose-response experiments (0.5–6 nM paclitaxel) for 24 h on proliferation (Fig. 1*a*) or for 6 h on apoptosis (Fig. 1*b*). Proliferation was assayed by two independent measures: by counting the number of trypsinized cells with a Burker Chamber (30) and by the ability of the cells to reduce MTT (30). The level of apoptosis was assayed by measuring the increase of cytoplasmic mono- and oligonucleosomes in an ELISA as described in the "Materials and Methods." Paclitaxel inhibited MDA-MB-435 proliferation, measured by either method, with a sigmoidal

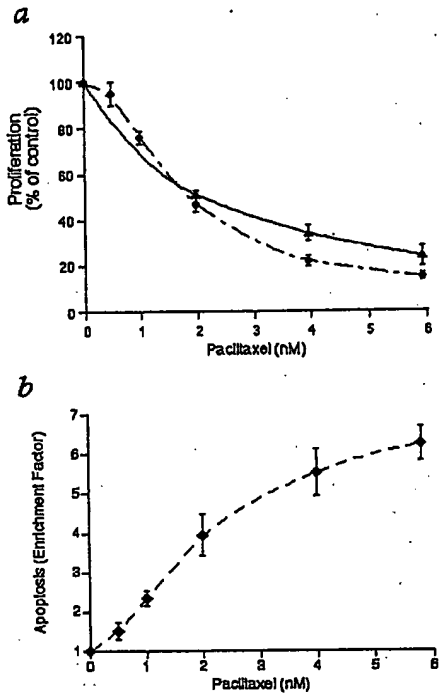

Fig. 1 Paclitaxel treatment induces apoptosis in MDA-MB-435 cells. Cell monolayers were treated with increasing concentrations (0–6 nM) of paclitaxel and (*a*) cell number was measured by either cell counting in a Burker Chamber (●) or MTT analysis (▲). *b*, apoptosis was examined by determining nucleosomal DNA fragmentation using an apoptosis determination kit. Rate of apoptosis is reflected by the enrichment of nucleosomes in the cytoplasm shown on the Y-axis. Data represent the mean of four different experiments, each performed in triplicate; *bars*, ±SE.

dose-effect relationship, and calculated IC_{50} values of 2.3 ± 0.5 and 2.1 ± 0.3 nM for cell number (Fig. 1, *circles*) and MTT (Fig. 1, *triangles*), respectively. The kinetics of paclitaxel-induced apoptosis (Fig. 1*b*) were similar, having a sigmoidal increase in apoptotically produced cytoplasmic nucleosomes in a paclitaxel dose-dependent manner with a calculated IC_{50} of 1.8 ± 0.2 nM paclitaxel.

Paclitaxel Inhibits Na^+/H^+ Exchange Activity, and Stimulates p38 MAP Kinase and PKA Activity. We next assessed the effect of paclitaxel on the activity and expression of the Na^+/H^+ exchanger in MDA-MB-435 cells. Na^+/H^+ exchange activity was measured as the recovery in pHi with the fluorescent probe 2′, 7′-bis(carboxyethyl)-5(6)-(carboxyfluorescein pentaacetoxymethy) ester after an induced acid load. Cells were treated with different paclitaxel concentrations (0.5–6 nM) for 6 h, and pHi recoveries were measured after cells were acidified by a 5-min pulse of 20 mM NH_4Cl and then alkalinized by perfusion with a HEPES-NMEG Ringer at pH 7.4 (Fig. 2*a*). Each trace begins at the start of perfusion of the monolayer with HEPES-*N*-methyl-D-glucamine Ringer. After reaching a stable acidic pHi level, there was no recovery of pHi until the monolayer was perfused with sodium Ringer at which point a rapid recovery begins that corresponds to the activity of the NHE. Paclitaxel treatment had a dose-dependent inhibitory effect on Na^+/H^+ exchange activity. Fig. 2*b* summarizes this dose-dependent paclitaxel-induced inhibition of NHE activity, which occurred with a calculated IC_{50} of 1.7 ± 0.4 nM paclitaxel, a value not significantly different from the IC_{50} for paclitaxel-dependent apoptosis ($P = 0.042$). To determine whether the change in Na^+/H^+ exchange activity derived from a decreased expression of NHE1 or to a decreased turnover rate per transporter, we analyzed NHE1 expression by Western blot in nontreated cells and cells treated with different concentrations of paclitaxel (1 nM, 2 nM, and 4 nM) for 6 and 24 h (Fig. 2*c*). Paclitaxel treatment did not change NHE1 protein expression, demonstrating that the paclitaxel inhibitory effect on NHE1 activity is not associated with the modulation of its expression.

As paclitaxel has been shown to alter the long-term activity and phosphorylation state of all three of the MAP kinases in complex patterns depending on cell/tumor type (10–16), we first determined the effect of a 24-h treatment of 4 nM paclitaxel on the activities of p38, c-Jun NH_2-terminal kinase, and extracellular signal-regulated kinase using two different antibodies: a phosphospecific antibody and an antibody that recognizes total protein expression. This treatment had no effect on either extracellular signal-regulated kinase or c-Jun NH_2-terminal kinase phosphorylation state, while inducing a strong increase in p38 phosphorylation state (data not shown). We then performed a dose and time course experiment for the activation of p38 by paclitaxel to better characterize the dynamics of its activation (Fig. 2*d*). At 4 h of treatment, activation of p38 was observed only at 2 and 6 nM paclitaxel, whereas at 24 h paclitaxel treatment activated p38 at all of the concentrations. The total expression of p38 remained unaltered in all of the treatments.

PKA has been reported to be rapidly activated by paclitaxel in MCF-7 breast cancer cells (6). Therefore, we next examined whether changes in the catalytic activity of PKA occur during paclitaxel treatment (Fig. 2*e*). Cells were treated for 30 min with the indicated paclitaxel concentrations, and the ability of a cytosolic extract to phosphorylate a PKA-specific peptide in the presence and absence of cyclic AMP was measured via an ELISA assay. Stimulation of PKA activity by Fsk (1 μM), the pharmacological activator of adenylate cyclase, was used as a positive control. As was observed for p38, PKA was stimulated in a dose-dependent manner by a range of paclitaxel concentrations similar to that which is stimulatory for apoptosis.

Role of p38 MAP Kinase and PKA in Paclitaxel-induced Apoptosis, and Inhibition of Na^+/H^+ Activity. We next investigated the role of p38 and PKA in paclitaxel-dependent stimulation of apoptosis and inhibition of NHE1 activity using activators or inhibitors of p38 and PKA together with 0.5 or 4 nM paclitaxel, respectively. To inhibit p38 MAP kinase cells were either incubated with SB203580 (100 nM) or transiently transfected with 10 μg of plasmid containing a mutated sequence of the α-isoform of p38 MAP kinase (K>M) that inhibits its action. To activate p38 the cells were transiently transfected with 10 μg of plasmid containing the constitutively active upstream activator of p38, MKK6(E). The effect of modulating PKA activity was evaluated by treating cells with the PKA-specific inhibitor, H89 (100 nM), or Fsk (1 μM). Apoptosis was measured by the increase in cytoplasmic DNA-histone complex in cells incubated for 6 h with the respective paclitaxel concentration ± the various kinase activators or in-

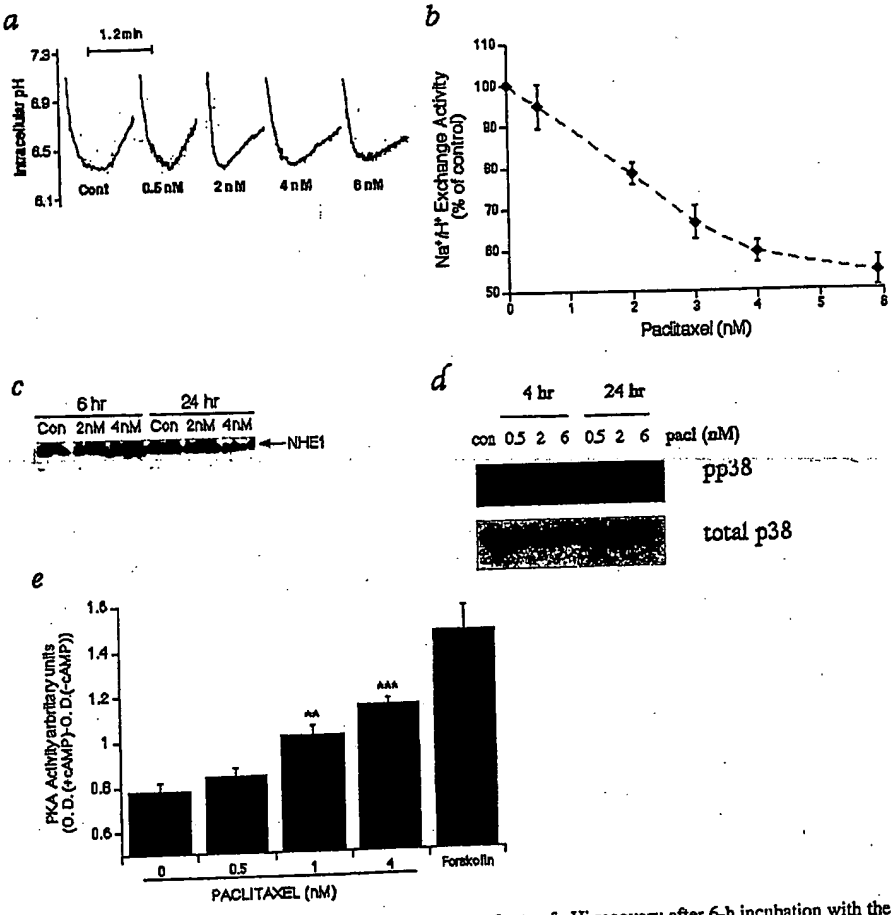

Fig. 2 Effect of paclitaxel on NHE1, p38, and PKA. *a*, typical experiments of rate of pHi recovery after 6-h incubation with the indicated paclitaxel concentration. Each trace begins at the start of perfusion of the monolayer with HEPES-NMEG solution (pH 7.4). After reaching a stable pHi, there was no recovery of pHi under this condition. When the monolayer was perfused with 135 mM Na^+ nominally bicarbonate free-HEPES solution (pH 7.4) a rapid recovery of pHi commenced. The recovery in a control monolayer is compared with that in a series of monolayers incubated with increasing paclitaxel concentrations measured at 135 mM sodium. *b*, pHi recoveries were measured as above, and the initial rate of the NHE activity calculated as Na^+-dependent H^+ efflux as described in "Materials and Methods." Data represent the mean ($n = 20$); *bars*, ±SE. *c*, Western Blot analysis of NHE1 expression after treatment of monolayers with paclitaxel for the indicated time and concentration. *d*, the phosphorylation state of p38 or (*e*) the activity of PKA were measured after incubation with indicated concentrations for the indicated times as described in "Materials and Methods." Data presented for the phosphorylation state of p38 show one representative of three independently performed experiments whereas PKA data are the mean of between 6 and 10 observations for each condition; *bars*, ±SE. ** $P < 0.01$, *** $P < 0.001$ compared with paclitaxel-treated cells at the same paclitaxel concentration.

hibitors (Fig. 3*a*). Blocking the activity of either PKA or p38 greatly reversed the stimulation of apoptosis by 4 nM paclitaxel, whereas, on the contrary, activating either kinase during paclitaxel treatment resulted in an increase of the effect of 0.5 nM paclitaxel on apoptosis. These results suggest that p38 and PKA activation precede paclitaxel-dependent apoptosis and form part of its mechanism of action.

To assay the involvement of p38 and PKA in the inhibition of NHE1 induced by paclitaxel, the same pharmacological agents and plasmids as above were used, and cells were treated as described for NHE activity measurements (Fig. 3*b*). As observed for the induction of apoptosis by paclitaxel, inhibition of either PKA or p38 reversed the paclitaxel-dependent inhibition of NHE activity, whereas stimulation of either kinase produced an increase in the paclitaxel inhibitory effect on NHE activity. In basal conditions stimulating or inhibiting either PKA or p38 produced only small changes in NHE1 activity suggesting that in these cells PKA and p38 play only a small role in the maintenance of basal NHE1 activity levels (data not shown).

Altogether, these data suggest that PKA and p38 are upstream of NHE1 in the paclitaxel-dependent induction of apoptosis. Simultaneous incubation with PKA and p38 inhibitors or activators did not additively reverse or potentiate either apoptosis or NHE1 activity (data not shown), suggesting that they are part of the same pathway. To determine the sequence of this putative cascade, we used the above PKA and p38 inhibitors and activators together in the apoptosis assay such that an activator of one kinase preceded an inhibitor of the other kinase (Fig. 4*a*).

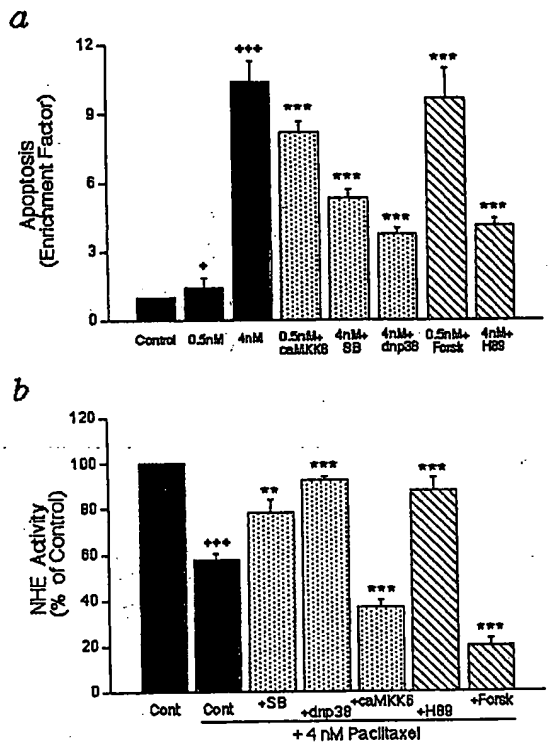

Fig. 3 PKA and p38 positively regulate paclitaxel induction of apoptosis and inhibition of NHE1. To examine the role of PKA (▨) and p38 (▤) in the paclitaxel-dependent activation of apoptosis and inhibition of NHE activity, MDA-MB-435 monolayers were exposed to either 0.5 or 4 nM paclitaxel after the cells had either been transfected with KRSPA-dnp38α (K>M) or KRSPA-caMKK6 (E) vectors encoding a dn mutant of the subunit of p38α or constitutively active upstream activator kinase of p38, respectively, or treated with pharmacological activators (Fsk for PKA) or inhibitors (H89 for PKA and SB203580 for p38). *a*, apoptosis was examined by determining nucleosomal DNA fragmentation or (*b*) NHE1 activity measured as in Fig. 2. Data are the mean of between 8 and 15 observations for each condition; *bars*, ±SE. + $P < 0.05$, +++ $P < 0.001$ compared with nontreated control cells; ** $P < 0.01$, *** $P < 0.001$ compared with paclitaxel-treated cells at the same paclitaxel concentration.

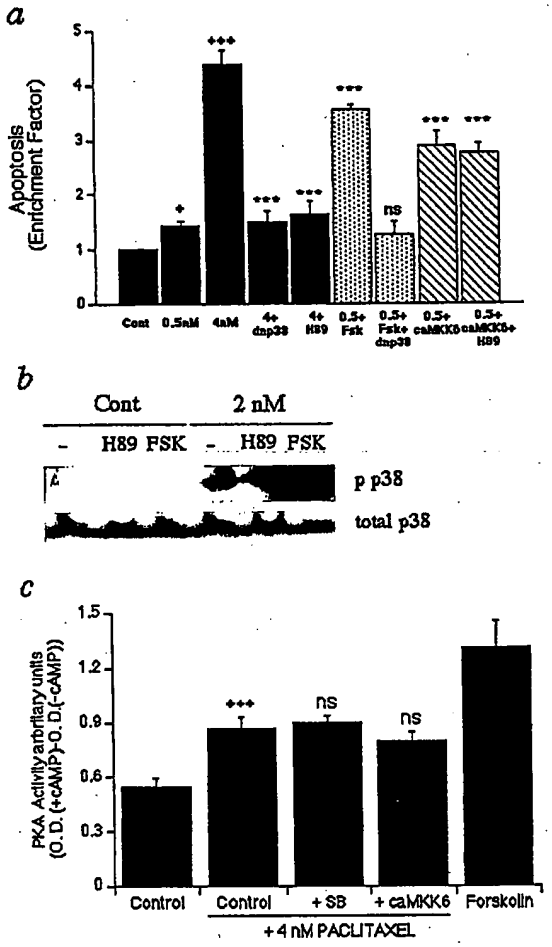

Fig. 4 PKA is upstream of p38 in the regulation of paclitaxel-dependent apoptosis. *a*, PKA-dependent potentiation of 0.5 nM paclitaxel-induced apoptosis is blocked by inhibiting p38 (via SB or dnp38α), whereas p38-dependent potentiation of 0.5 nM paclitaxel-induced apoptosis is not blocked by inhibiting PKA (via H89). Data are the mean of between 5 and 8 observations for each condition; *bars*, ±SE. *b*, paclitaxel-induced activation of p38 is dependent on PKA. The inhibition of PKA with H89 reduced, whereas stimulation of PKA with Fsk potentiated the increase in p38 phosphorylation after paclitaxel treatment. *c*, paclitaxel-induced activation of PKA is independent of p38. + $P < 0.05$, +++ $P < 0.001$ compared with nontreated control cells; *** $P < 0.001$ compared with paclitaxel-treated cells at the same paclitaxel concentration.

Whereas inhibition of p38 activity by dnp38α (or by SB203580; data not shown) was able to reverse the Fsk potentiation of 0.5 nM paclitaxel, the inhibition of PKA by H89 was not able to reverse the potentiation of 0.5 nM paclitaxel by caMKK6. These data strongly suggest that PKA is upstream of p38 in the regulatory apoptotic mechanism of paclitaxel. This conclusion is additionally supported by the ability of H89 to block and Fsk to potentiate paclitaxel-dependent phosphorylation of p38 by 2 nM paclitaxel (Fig. 4*b*), whereas neither inhibition of p38 with SB203580 nor its stimulation with caMKK6 had any effect on paclitaxel-dependent activation of PKA (Fig. 4*c*):

Inhibition of the NHE1 Participates in and Potentiates Paclitaxel-induced Apoptosis. To define the role of NHE in paclitaxel-dependent stimulation of apoptosis, we analyzed the effect of the specific inhibition of the NHE1 by the amiloride analogue, DMA, on paclitaxel-dependent apoptosis. If the inhibition of NHE1 is an integral part of the mechanism by which paclitaxel induces apoptosis, pharmacological inhibition of the NHE1 should potentiate paclitaxel action synergistically at low paclitaxel concentrations, and this potentiation should disappear as the paclitaxel concentration increases because its inhibition by paclitaxel reduces the possibility of additional inhibition by DMA. Cells were grown to confluence and treated for 6 h with different concentrations of paclitaxel (0.5–6 nM) in the presence (solid bars) or absence (open bars) of 2 μM DMA and apoptosis measured as above (Fig. 5). Incubation with DMA or 0.5 nM paclitaxel alone resulted in a small increase in apoptosis but

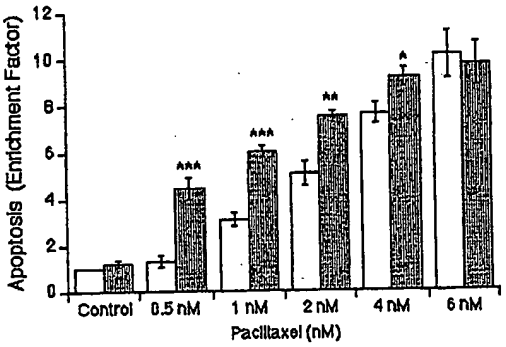

Fig. 5 Inhibition of NHE1 activity acts synergistically with paclitaxel to induce apoptosis in MDA-MB-435 cells. To examine the role of the NHE1 in paclitaxel-induced apoptosis, monolayers were treated with increasing concentrations (0–6 nM) of paclitaxel in the absence (□) or presence (■) of 2 μM DMA. At the end of 6-h incubation, the cells were extracted, and apoptosis was examined by determining nucleosomal DNA fragmentation as above. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with paclitaxel-treated cells at the same paclitaxel concentration.

Table 1 Effect of coinhibition of NHE activity on the IC_{50} of the paclitaxel-dependent induction of apoptosis in human breast cancer cells

Confluent cell monolayers were treated with increasing concentrations of paclitaxel for 6 h in the presence or absence of 2 μM DMA and apoptosis measured as described in "Materials and Methods." The value of the IC_{50} for paclitaxel-induced apoptosis in each experiment was calculated using the XLfit-3 program from ID Business Solutions (Emeryville, CA). Values are mean ± SE for IC_{50} (nM). Values in parentheses are the number of experiments and the fold decrease in paclitaxel IC_{50} values with DMA treatment. Significance is between the difference of the values in the presence and absence of 2 μM DMA.

	IC_{50} without DMA	IC_{50} with DMA
MDA-MB-435	2.14 ± 0.19	0.64 ± 0.09[a] (n = 5, 3.3X)
MCF-7	31.8 ± 0.49	13.2 ± 0.16[a] (n = 5, 2.4X)
SKBR3	92.2 ± 3.02	20.6 ± 0.42[a] (n = 4, 4.5X)

[a] $P < 0.001$.

when incubated together produced a significant, synergistic increase in apoptosis. DMA reduced the IC_{50} of paclitaxel-induction of apoptosis from ~2 nM to ~0.65 nM (Table 1). This synergistic potentiation of the sensitivity of cells to paclitaxel at lower paclitaxel concentrations was reduced with increasing paclitaxel concentration such that by 6 nM paclitaxel there was no additional potentiation of its apoptotic action by inhibition of the NHE1. This reduction in the ability of DMA to potentiate paclitaxel action at near maximal paclitaxel concentrations supports the hypothesis that in MDA-MB-435 cells the inhibition of NHE1 plays a fundamental role in paclitaxel induction of apoptosis. Paclitaxel is starting to be used in adjuvant therapy of nonmetastatic breast tumors (38). For this reason and to determine the generality of the role of the NHE1 in paclitaxel-induced apoptosis, these measurements were conducted in two other breast cancer cell lines having lower metastatic potential, MCF-7 (35) and SKBR3 (36). We first measured the NHE

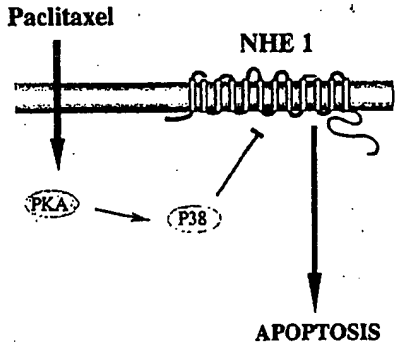

Fig. 6 Model of paclitaxel induction of apoptosis in breast cancer cells.

activity and its sensitivity to DMA in these two cell lines. In MCF-7 cells the mean activity of the NHE was 0.68 ± 0.09 ΔpHi/min and 2 μM DMA inhibited NHE activity by 84.2% ± 4% (n = 7), whereas in SKBR3 cells the mean activity of the NHE was 0.93 ± 0.13 ΔpHi/min and 2 μM DMA inhibited NHE activity by 76.2% ± 8% (n = 5). We had determined previously in cell number assays that these cell lines are less sensitive than MDA-MB-435 to paclitaxel (paclitaxel IC_{50} on cell growth of ~30 and 100 nM in MCF-7 and SKBR3, respectively). As can be seen in Table 1, in both cell lines the IC_{50} for paclitaxel-induced apoptosis was similar to that for cell growth and, most importantly, specific inhibition of the NHE1 resulted in a 3–4-fold decrease in the IC_{50} of paclitaxel-dependent apoptosis in both cell lines.

DISCUSSION

In this study, we describe a signal transduction cassette involved in regulating paclitaxel apoptosis in MDA-MB-435 cells. (Fig. 6). Our findings provide direct evidence demonstrating that PKA, p38, and NHE1 are involved in the paclitaxel-driven apoptotic process. Blocking either PKA or p38 drastically reduced the ability of paclitaxel to inhibit the NHE1 and to produce apoptosis, whereas stimulation of either kinase greatly potentiated apoptosis and inhibition of NHE1 even at low paclitaxel concentrations (Fig. 3, *a* and *b*). Furthermore, we provide evidence supporting the notion that these proteins are components of a single signaling cascade consisting sequentially of PKA, p38, and NHE1. That PKA lies upstream of p38 in regulating paclitaxel action was demonstrated by experiments in which inhibiting p38 blocked the Fsk-dependent potentiation of low-dose paclitaxel-induced apoptosis and stimulating p38 blocked the H89-dependent abrogation of apoptosis produced by high concentrations of paclitaxel. Conversely, the modulation of PKA activity had no effect on the p38-dependent effect (Fig. 4*a*). Additionally, agents that increased or inhibited PKA activity stimulated or reduced, respectively, the paclitaxel-dependent activation of p38, whereas agents that altered p38 activity had no effect on paclitaxel-dependent PKA activation (Fig. 4, *b* and *c*). Interestingly, our results unify recent, seemingly contradictory observations concerning the signal transduction mechanisms underlying paclitaxel action in the breast tumor MCF-7 cell line in which paclitaxel-induced Bcl-2 phos-

phorylation was mediated by activation of either PKA (6) or by activation of p38 (16). Together with those papers, our results suggest that PKA and p38 form part of the same signal transduction module in both ER(+) MCF-7 cells and ER(−) MDA-MB-435 cells, and that this common module regulates NHE1, Bcl-2 action, and consequent apoptosis. The questions concerning the common involvement of these kinases in paclitaxel-driven Bcl-2 phosphorylation in the MDA-MB-435 cell line and of the involvement of NHE1 in this process in both cell lines are currently under investigation in our laboratory. It is probable that there are kinases both up-stream and down-stream of PKA and p38 that participate in the orchestration of the paclitaxel-induced apoptotic response. The identification and characterization of these potential kinases will be investigated in our laboratory.

One of the most significant findings of the present study was the observation of the involvement of the Na^+/H^+ exchanger isoform NHE1 in the signal transduction mechanism driving paclitaxel-dependent apoptosis. Whereas the NHE1 has been shown to participate in regulating the response to other proapoptotic substances (17–20), our findings provide the first evidence demonstrating that NHE1 is involved in paclitaxel-dependent apoptosis (Fig. 3*b*; Fig. 5; Table 1). Importantly, paclitaxel-dependent apoptosis was synergistically increased at low paclitaxel concentrations by the contemporary exposure to DMA, a specific and potent pharmacological inhibitor of the NHE1 (Fig. 5; Table 1), demonstrating a direct role of NHE1 in paclitaxel-driven apoptosis. NHE1 plays a pivotal role in mediating tissue injury during ischemia and reperfusion, and, consequentially, a clinical protocol has been defined providing a rationale of a large-scale Phase II/Phase III trials designed to evaluate the safety and efficacy of specific inhibitors of the exchanger (32–34). The very low toxicity of NHE1 inhibitors observed in these clinical trials together with the high basal NHE1 activity found in tumors (39) should facilitate the transition from the described theoretical framework to the implementation of novel therapies enhancing the efficacy of paclitaxel. On the basis of our data there is a rationale for the rapid inclusion of this NHE1 inhibitor in clinical trials with paclitaxel.

ACKNOWLEDGMENTS

We thank Dr. Stephan Ludwig of the University of Würzburg, Würzburg, Germany, for his kind gift of the KRSPA plasmids for dnp38 and caMKK6.

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